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A Recombinase-Aided Amplification Assay for the Detection of *Chlamydia felis*

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Abstract

Chlamydia felis is an important zoonotic agent for humans and various animals. A recombinase-aided amplification (RAA) assay was developed for detecting *C. felis*. RAA can be performed in a closed tube at 39°C within 30 min. The detection limit was 10.6 copies of the *C. felis* plasmid DNA per reaction. No positive signals for other pathogens were detected. The coincidence rate of RAA and conventional PCR was 95.24% (20/21) and 100% (96/96) for positive and negative samples, respectively. The established RAA assay is a simple, rapid, highly sensitive, and specific method for detecting *C. felis*.

Keywords: *Chlamydia felis*, recombinase-aided amplification, rapid detection

The family *Chlamydiaceae* (order Chlamydiales, phylum Chlamydiae) contains obligate intracellular Gram-negative bacteria, which are pathogenic to humans and animals (Horn 2008). The family *Chlamydiaceae* comprises one genus, *Chlamydia* (Sachse et al. 2015; Pannekoek et al. 2016). The *Chlamydia* genus is composed of several species, including *C. trachomatis*, *C. muridarum*, *C. suis*, *C. abortus*, *C. pecorum*, *C. pneumoniae*, *C. psittaci*, *C. caviae*, and *C. felis* (Sachse et al. 2015). *C. felis* is a zoonotic agent and a pathogen adapted to cat family members causing severe conjunctivitis associated with upper respiratory tract diseases (Rampazzo et al. 2003; Rodolakis et al. 2010). *C. felis* is common among cats living in shelters, especially in facilities with high population density, particularly if the shelters have unscheduled disinfection and inadequate ventilation (Bannasch and Foley 2005). In addition, symptomatic cats with *C. felis*-positive test results without effective environmental decontamination and veterinary treatments may deteriorate clinically or even die (Gonsales et al. 2016).

The current detection methods for *C. felis* include isolation and identification of the pathogen, enzyme-linked immunosorbent assay (ELISA), and PCR assay (Clarkson and Philips 1997; Helps et al. 2001; Ohya et al. 2010; Wons et al. 2017; Barimani et al. 2019) However, these methods need sophisticated equipment for a PCR assay and a relatively lengthy readout time for ELISA. Thus, there is an urgent need for a simple and faster detection method. Recombinase-aided amplification (RAA) using specific enzymes is a newer isothermal amplification technology that can detect pathogens quickly. It is more convenient than PCR because it can be conducted outside a lab environment with a constant reaction temperature (37–42°C) (James and Macdonald 2015). In addition, the RAA-amplified product of the target gene can reach detectable levels within 5–20 min after the reaction has commenced (Wu et al. 2021). This method has been widely explored to detect human and animal pathogens, such as Wuxiang virus (Yao et al. 2022) and SARS-CoV-2 (Xue et al. 2020). This study aimed to establish a rapid RAA assay to detect *C. felis* and then evaluate the assay using clinical samples from stray cats.

The target segment (position 752–981 in the 16S rRNA of the Fe/Pn-1 *C. felis* strain [NR_036876.1])

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Table I
Sequences of primers and probes in the RAA assay for *Chlamydia felis*.*

Primers/Probes	Sequence (5'–3')	Genomic position†
C. felis F	GAAAGCAAGGGGAGCAAACAGGATT	769–793
C. felis R	TCAGGCGGCATACTTAACGCGTTAG	891–867
C. felis probe	CGATGCATACTTGATGTGGATAGTCTCAACCC[FAM-dT] A(THF)[BHQ1-dT] CCGTGTCGTAGCTAAC(C3-Spacer)	821–871

* – BHQ – black hole quencher, C3-spacer – 3' phosphate blocker, FAM – 6-carboxyfluorescein, THF – tetrahydrofuran
† – the primer and probe locations in the genome were based on the 16S rRNA sequence of Fe/Pn-1 *Chlamydia felis* strain (GenBank Access No.: NR_036876.1)

was synthesized chemically and inserted into the cloning vector pUC57 to construct recombinant vector *C. felis*_pUC57. The concentration of *C. felis*_pUC57 was determined using an ultra-micro spectrophotometer (Thermo Fisher Scientific Inc., USA), and then the copy number was calculated. RAA-specific primers and a probe were designed and produced based on the 16S rRNA segment of *C. felis* (Table I). The length of the amplification product was estimated to be 123 bp (position 769–891 in the 16S rRNA gene of *C. felis*).

The RAA reaction was performed using an RAA nucleic acid amplification kit (Jiangsu Qitian Biotechnology Co., Ltd., China), which contained an enzyme mixture (including DNA polymerase, 30 ng/μl; DNA-binding protein (SSB), 800 ng/μl; and recombinase UvsX, 120 ng/μl) required for DNA amplification in the lyophilized form in a single tube. The following substances were present in the reaction mixture: 25 μl of rehydration buffer, 0.6 μl of *C. felis* probe (10 μmol/l), 2.1 μl of each primer (10 μmol/l), 13.2 μl of double distilled water (ddH_2O), and 2 μl of the DNA template. Afterward, 45 μl of the master mixture/template solution was mixed and transferred to the lyophilized enzyme mixture. Each tube lid was then pipetted with 5 μl of 0.28 M magnesium acetate. Before initiating the RAA reaction, the tube lids were closed, vortexed, and centrifuged. The *C. felis*_pUC57 was 10-fold serially diluted for the analytical sensitivity assay from 1.06×10^7 copies/reaction to 1.06×10^0 copies/reaction. The RAA reaction was carried out in a simple incubator for 30 min at 39°C, using a portable UMG-1600 thermostatic fluorescence detector (Hangzhou UMI Instruments Co., Ltd., China), and the fluorescence signals were collected every 10 seconds. Each run included a negative control of ddH_2O. To confirm the specificity of the RAA assay, it was tested using a concentration of 1×10^5 copies/reaction of nucleic acids from feline parvovirus/Shanghai/01/2021(FPV), feline herpesvirus-1/Cat/Shanghai/01/2014 (FHV-1), feline calicivirus-SH202101 (FCV). The viral nucleic acids were isolated in the animal rescue in Shanghai by the Shanghai Animal Disease Prevention and Control Center. Feline infectious peritonitis virus VR-990 (FIPV), *Mycoplasma felis* ATCC® 23391™, and *Bordetella bronchiseptica* ATCC® 4617™ were bought from the American Type Culture Collection (ATCC®, USA), respectively, and used throughout the experiments. The consistency between the RAA and the real-time PCR assay was evaluated on 117 eye, nasal, and oropharynx swabs. The real-time PCR reaction was performed using a *C. felis* real-time PCR detection kit (Shanghai Erchuang Biotechnology Co., Ltd, China), developed with slight modifications as previously described (Helps et al. 2001). The reaction mixture contained 12.5 μl of 2 × qPCR buffer, 1 μl of TaKaRa Ex Taq® HS enzyme (5 U/μl), 0.5 μl of 10 μM primer mix in the same proportion (Forward primer: 5'-ATGCTTGTTCCATACATTGGGG-3'; Reverse primer: 5'-TCCTAAAAGAGTTGGGTTCCAGG-3'), 0.5 μl of 10 μM probe (5'-FAM-CGGCGACACTATCCGCATTGCTCAACCGCCG-BHQ-3'), 8.5 μl of ddH_2O, and 2 μl of template. It was performed using the ViiA 7 Real-Time PCR system (Thermo Fisher Scientific Inc., USA) per the manufacturer's instructions at 94°C for 2 min, followed by 40 amplification cycles of 10 s at 94°C and 60 s at 60°C.

The RAA assay was positive for *C. felis* but negative for the other pathogens tested (Fig. 1A). The sensitivity of the RAA assay was examined using a dilution gradient of the *C. felis*_pUC57 plasmid ranging from 1.06×10^7 copies/reaction to 1.06×10^0 copies/reation. As presented in Fig.1B, the RAA-mediated fluorescence signal could be detected when the concentration of the plasmid DNA was greater than or equal to 1.06×10^1 copies/reaction. Moreover, the higher the template concentration, the higher the fluorescence value and earlier the peak time could be observed (Fig. 1B).

In this study, one swab per cat was collected from the eye, the nasal, or the oropharynx presenting severe clinical signs, and 117 cats that were suspected of having *C. felis*-related disease between July 2019 and October 2021 were included. Specifically, 24 eye, 36 nasal, and 57 oropharynx swabs from stray cats were tested

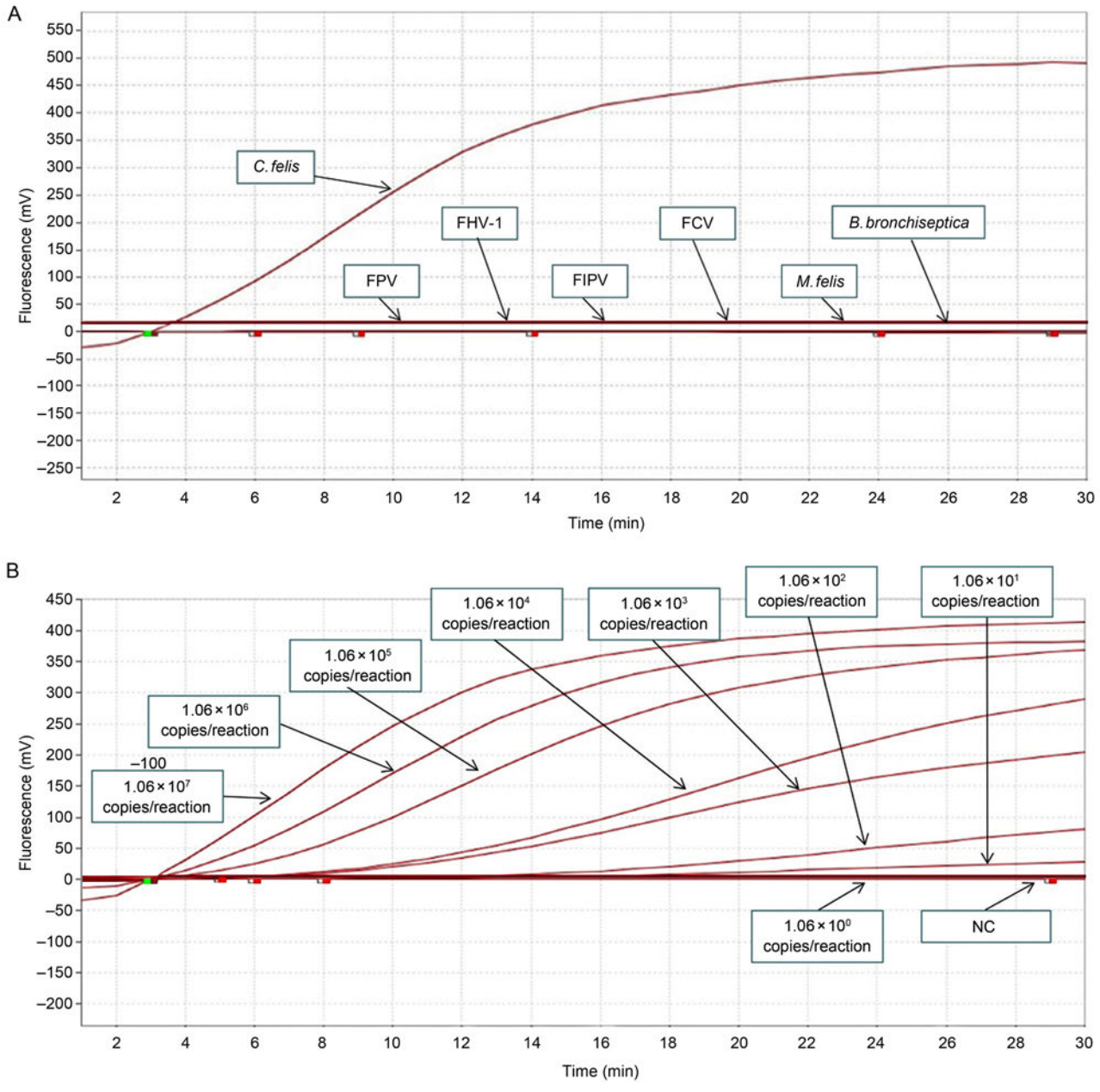


Fig 1. A) Specificity evaluation of the recombinase-aided amplification (RAA) assay for the detection of *Chlamydia felis* using genomic DNA from *C. felis*_pUC57 plasmid, feline calicivirus-SH202101 (FCV), feline herpesvirus-1/Cat/Shanghai/01/2014 (FHV-1), feline infectious peritonitis virus VR-990 (FIPV), feline parvovirus/Shanghai/01/2021 (FPV), *Mycoplasma felis* ATCC® 23391™ (*M. felis*), and *Bordetella bronchiseptica* ATCC® 4617™ (*B. bronchiseptica*), as the templates of RAA, respectively.
B) Examination of the RAA assay's sensitivity for detecting *Chlamydia felis* using a serial dilution of the *C. felis*_pUC57 plasmid as the template. The lower limit of detection was 10.6 copies/reaction plasmid DNA. The higher the template concentration, the greater the fluorescence value and the earlier the peak time. NC, negative control group. All the experiments were performed at least three times.

in RAA and real-time PCR assay. A total of 21 samples, including four eyes, nine nasal, and eight oropharynx swabs, were positive in real-time PCR. In contrast, the RAA assay produced positive results in 20 of the 21 positive samples in real-time PCR (Table II), with one nasal test sample with an inconsistent result, showing a positive predictive agreement of 95.24% between the RAA assay and real-time PCR. The RAA assay yielded a negative result in all 96 negative samples that did not show amplification in real-time PCR, providing a predictive agreement of 100% between the RAA assay and real-time PCR.

In the last three decades, PCR has been successfully used to detect and/or identify infectious pathogens, including a real-time PCR for detecting *C. felis* (Helps et al. 2001). However, the field application of PCR-based assays is poorly implemented in resource-limited areas due to the requirement of sophisticated equipment. Recently, RAA has been extensively explored for the detection of human and animal pathogens since

Table II
Comparison of *Chlamydia felis* detection in 117 swabs examined by RAA and real-time PCR.

		Real-time PCR		Performance characteristics	
		Positive	Negative	Sensitivity	Specificity
RAA	Positive	20	0	95.24%	100%
	Negative	1	96		
	Total	21	96		

the reaction proceeds at a relatively low and constant temperature without the need of complex equipment (Xue et al. 2020; Wu et al. 2021; Yao et al. 2022).

Here, a newer molecular method for rapidly detecting the 16S rRNA of *C. felis* was developed using RAA. Because the RAA assay can be conducted at 39°C, and scanned using a portable real-time fluorescence device, it is highly relevant for the molecular detection of *C. felis* infection in veterinary clinics and hospitals, especially in low-equipment environments. The RAA assay for *C. felis* had a limit of detection of 10.6 copies per reaction in the present study. No cross-reactions with FCV, FHV-1, FIPV, FPV, *M. felis* ATCC® 23391™, and *B. bronchiseptica* ATCC® 4617™ were observed, demonstrating the robust specificity of the assay. Compared to real-time PCR, the RAA assay had a sensitivity of 95.24% and a specificity of 100%. Clinical sample analysis revealed that one of the 21 real-time PCR-positive samples was detected as a negative in the RAA assay. It might have occurred as the viral load in the sample was lower than the RAA detection limit. The major limitation of this study is throughput when the RAA assay is used in a field or clinical setting because the portable detector can only detect eight samples in a single run.

The percentages of stray cats positive for *C. felis* infection have been reported to be 14.7% in Britain (McDonald et al. 1998), 20.0% in Italy (Rampazzo et al. 2003), and 11.5% in Switzerland (von Bomhard et al. 2003),15.3% in Sweden (Holst et al. 2006), and 4.6% in America (Low et al. 2007). In Japan, 28.9% of domestic cats and 26.3% of stray cats were reported to be positive for *C. felis* infection (Cai et al. 2002). Moreover, *Chlamydia* species can infect humans and seriously impact public health because they can cause coronary heart disease, atherosclerosis, pneumonia, and other serious diseases (Carlisle and Nahata 1999; Larsen et al. 2002; Grayston 2005). Therefore, a rapid and simple detection method for *C. felis* is an essential tool to confirm the transmission of this pathogen in cats potentially.

In summary, a rapid, highly sensitive, and specific RAA method was exploited to detect the 16S rRNA gene of *C. felis*. This assay can provide a potentially halpful substitute for qPCR for detecting *C. felis* in low-resource settings.

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Author contributions

Conceptualization: Jian Liu, Hongjin Zhao, Weidong Qian; Methodology: Jian Liu, Yaping Gui, Luming Xia, Guohua Gong, Feifei Ge; Formal analysis and investigation: Jian Wang, Yilan Bai; Writing-original draft preparation: Weidong Qian, Haixiao Shen, Xiaojing Chang; Writing-review and editing: Weidong Qian, Jian Liu; Funding acquisition: Hongjin Zhao; Resources: Yilan Bai; Supervision: Jian Wang. All authors contributed to manuscript revision and approved the final version.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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